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4. Title of the invention

DELIVERY OF NITRIC OXIDE II

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Cruikshank & Fairweather
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DELIVERY OF NITRIC OXIDE II

FIELD OF INVENTION

The present invention relates to zeolites containing releasably adsorbed nitric oxide, methods of preparing these zeolites, methods of releasing the nitric oxide into a solution or into air, and uses thereof.

BACKGROUND OF THE INVENTION

Nitric oxide (the chemical formula is NO) is a remarkable small molecule that is vitally important in many biological processes. It is a vasodilator that increases blood flow through arteries and veins, and is also an important factor in controlling platelet adhesion and aggregation. It also plays a crucial role in the immune system. Much is now known about the mode of action of nitric oxide and it is clear that it has enormous potential in medicine and biotechnology in both *in vivo* and *ex vivo* applications.

The controlled delivery of nitric oxide may be important in therapy. For example, nitric oxide can prevent thrombosis and restenosis following balloon angioplasty and stent insertion in blocked arteries (International Patent Application WO 95/24908). The delivery of nitric oxide to the skin may also have therapeutic benefits for patients with peripheral circulatory problems which can occur in conditions such as arthritis and Raynaud's syndrome. Nitric oxide also plays a part in wound healing and angiogenesis, and delivery of nitric oxide to wounds can be beneficial when healing is slow which can occur, for example, in elderly patients (M. Shabani et al, Enhancement of wound repair with a topically applied nitric oxide-releasing polymer *Wound repair and regeneration*, 4, 353, 1996 and S. Frank

H. Kampfer, C. Wetzler, J. Pfeilschifer, Nitric oxide drives skin repair: Novel functions of an established mediator *Kidney International*, 61, 882, 2002).

However the delivery of nitric oxide to the desired area, and in the required optimum dose is often difficult because nitric oxide is a gas. Delivery of nitric oxide is difficult in both *ex vivo* e.g. biotechnology applications and *in vivo* e.g. medical applications.

Various methods of nitric oxide delivery are known such as

(a) molecules which release NO spontaneously;
(b) molecules which are metabolised to give NO;
(c) molecules that release NO on photoactivation;
(d) release of NO from polymers and polymer coatings;
(e) production of NO from a chemical reaction.

The class (a) molecules are known as nitric oxide nucleophile complexes (NONOates) (C.M. Maragos et al, Complexes of NO with nucleophiles as agents for the controlled biological release of nitric-oxide-vasorelaxant effects *J. Med. Chem*, 34, 3242, 1991). These are a variety of molecules which give off nitric oxide spontaneously and have been shown to have a possible use in therapeutic applications (US Patent 4954526). However the use of NONOates in therapy is limited because they become distributed throughout the body which may compromise selectivity. The by-products following the release of NO may also form carcinogenic secondary nitrosamines.

The class (b) molecules include glyceryl trinitrate and sodium nitroprusside (L.J. Ignarro Biosynthesis and metabolism of endothelium-derived nitric-oxide *Ann. Rev. Pharmacol. Toxicol.* 30, 535, 1990). These compounds are

currently widely used as vasodilators, however prolonged use can lead to toxic side products such as cyanides. Furthermore, because these molecules need to be metabolised to release NO, the targeting of NO to particular sites may also be poor resulting in the effects tending to be systemic.

The class (c) molecules require specific activation, for example, light having a specific wavelength which can be difficult to initiate (C. Works, C.J. Jocher, G.D. Bart, X. Bu, P.C. Ford, Photochemical Nitric Oxide Precursors *Inorg. Chem.*, 41, 3728, 2002).

Class (d) release of nitric oxide mitigates the problems associated with systemic activity by delivering nitric oxide to a specific target site by supporting a nitric oxide releasing compound on a solid article. Such NO releasing compounds may be polymeric materials which can be coated onto medical instruments which can be used to target specific areas of the body for treatment. The polymers may contain, for example, the N_2O_2 group that releases NO after a chemical reaction (International Patent Application WO 95/24908 and US Patent Application 2002094985). However, the release of NO in such circumstances can be difficult to control and currently the preparation of the required materials may be expensive. The possible use of such polymers has been shown in the treatment of cardiovascular problems, for example, restenosis.

Class (e) delivery of nitric oxide has been proposed for topical applications by releasing nitric oxide from a chemical reaction. The chemical reaction involves the application of sodium nitrite, ascorbic acid and maleic acid, which gives off NO when contacted by water (US Patent Application No. 6,103,275). However, this reaction takes place only in acidic conditions and so may

cause irritation, especially to sensitive skin of elderly patients.

The object of the present invention is to obviate and/or mitigate the problems of nitric oxide storage and delivery.

SUMMARY OF THE INVENTION

According to a first aspect of the present invention there is provided a zeolite material comprising releasably adsorbed nitric oxide.

Zeolite materials are a class of aluminosilicate materials which are known and used in a number of applications, for example, ion exchange, gas separation and catalysis (A. Dyer, An Introduction to Zeolite Molecular Sieves, J. Wiley and Sons, 1988).

According to a second aspect of the present invention there is provided a method of preparing a zeolite material which comprises releasably adsorbed nitric oxide, said method comprising the steps of providing a zeolite material and contacting said material with nitric oxide gas.

Zeolites which are suitable for the present invention may be either naturally found or synthetically made. The zeolites contain pores and channels having dimensions which allow small molecules or ions to be adsorbed onto the internal surfaces of the material. The general formula of a zeolite framework is $Al_ySi_{1-y}O_4^{y-}$. For each aluminium atom in the zeolite framework, one negative charge is introduced which must be balanced by an extra-framework cation. These cations can be inorganic or organic in nature, and can be exchanged using standard ion exchange processes (M.E. Davis, Ordered porous materials for emerging applications *Nature* 417, 813, 2002).

The zeolites may comprise transition element cations as the extra-framework species e.g. iron, copper, ruthenium, and such zeolites can adsorb nitric oxide to form complexes inside the cavities of the zeolite material. These complexes are strong and may enable the nitric oxide to be stored until needed. Cations of other elements, for example, sodium and potassium bind nitric oxide less strongly. Those skilled in the art may use standard ion exchange processes to introduce the required metal ions into a zeolite structure as extra-framework cations (Plank et. al., U.S. Patent No. 3,140,249; Preparation, characterisation, and Performance of Fe-ZSM-5 Catalysts R. Joyner and M. Stockenhuber, J. Phys. Chem. B., 1999, 103, 5963-5976). Using such techniques it is possible to incorporate mixtures of cations in the zeolite structures.

The zeolites may be provided in a dehydrated state.

The amount of nitric oxide which may be loaded into the zeolites can be controlled by varying the relative amounts of the extra-framework cations, controlling their chemical nature, and/or the total number of ions present. For example, the number of extra framework cations present in the zeolite structure may depend on the amount of aluminium present in the framework. More aluminium ions require more extra framework cations to balance the negative charge. The extra framework cations may then interact with the NO molecules.

The chemical nature of the extra framework cations may also be changed (for example monovalent cations, e.g. Na^+ and Ag^+ may be exchanged for divalent cations, e.g. Fe^{2+} and Cu^{2+} or trivalent cations, e.g. Ru^{3+} and Fe^{3+}). Each different cation may have a different affinity for NO and changing the cations present in the zeolite framework may be used to control the release of NO. Such

manipulation of the zeolite composition can affect the rate at which the nitric oxide is released from the zeolite. For example a sodium-loaded zeolite may bind nitric oxide less strongly than an iron-loaded zeolite to release the nitric oxide more rapidly. A mixed sodium/iron zeolite may release nitric oxide at a different rate to either a sodium-loaded zeolite or an iron-loaded zeolite, and such release of nitric oxide may present a different rate profile.

The choice of zeolite framework can also be used to vary the loading and release rate of nitric oxide. Zeolite frameworks are available in synthetic materials with a variety of different structures, and suitable frameworks may be chosen that offer the desired properties for the application under consideration. For example, the pores and channels in a zeolite structure may be defined by the size of the pore or channel openings. The zeolite with the structure LTA has openings defined by 8 pore tetrahedral units (i.e. a ring of 8Si/Al atoms and 8 oxygen atoms). Zeolite MFI has a larger ring opening defined by 10 tetrahedral units, and FAU by an even larger pore opening of 12 tetrahedral units. The dimensionality of the pores can also differ between zeolite frameworks. For example, some zeolites have channels that run in only one direction (one dimensional channel systems) while others have systems of interacting channels in two or three dimensions (2-dimensional and 3-dimensional channel systems). The size, shape and dimensionality of zeolites may affect the rates of diffusion and adsorption/desorption of NO, and may be used to control the rate of release of NO from the zeolite in a particular application.

Thus, the composition of the zeolite material may be tailored to control the amount of nitric oxide loaded

into the zeolite structure and/or the rate at which the nitric oxide is released from the zeolite.

Such zeolite structures may be chosen from, but not limited to, frameworks having the following three letter framework codes: LTA, FAU, MFI, MOR, FER, BEA, PHI and SAS (See International Zeolite Association Website www.iza-online.org for details of how the codes relate to the frame-work structures of the zeolites which is incorporated herein by reference). These three letter codes describe the framework architecture of the zeolites, that is their structure, but do not describe the composition of the zeolite which may vary widely. The three letter codes are used as a nomenclature system for zeolites.

The zeolites which may be used in the present invention may have the following general formula (I):

$$\left[\left(M1^{n+}\right)_{x/n}\left(M2^{p+}\right)_{y/p}\ldots\left(Mn^{q+}\right)_{v/q}\right]\left[Al_zSi_{2-z}O_4\right] \qquad (I)$$

wherein M1 and M2 ... Mn are extra framework metal cations of elements selected from the group consisting of Li, Na, K, Ca, Mg, Fe, Cu, Ru, Rh, Co, Ni, Zn and Ag.
x may range from zero to nz,
y may range from zero to pz, and
v may range from zero to qz,
subject to the condition that $x/n + y/p + \ldots + v/q = z$.
z is the number of silicon atoms replaced by aluminium atoms in the zeolite framework.
n+, p+ and q+ are the charges of the extra framework metal cations, and may individually take the values of +1, +2 or +3.
M1 and M2 ... Mn may also be chosen from small organic cations such as $N(R_1)_a(R_2)_b^+$ wherein R_1 and R_2 are

independently selected from H, $-CH_3$, $-CH_2CH_3$, or $-CH_2CH_2CH_3$, and a and b are independently 0, 1, 2, 3 or 4 such that a + b = 4;

When M1 and/or M2 are small organic cations, NH^+_4 is prefered.

The zeolites which preferably may be used in the present invention have the following general formula (II):

$$[(M1^{n+})_{x/n}(M2^{p+})_{y/p}]\ [Al_zSi_{2-z}O_4] \qquad (II)$$

wherein M1 and M2 are as defined previously,
x may range from zero to nz, and
y may range from zero to pz, subject to the condition that $x/_n + y/_p = z$.

Prior to nitric oxide loading, the zeolites for use in the present invention may be fully or partially dehydrated, for example, under vacuum to remove water from the zeolite channels. The resulting zeolite may then be exposed to nitric oxide to load the zeolite.

Typically, the nitric oxide loading is performed at a temperature from -100°C to 50°C.

The loading of nitric oxide may be performed with pure NO or with a mixture of NO and a carrier gas such as an inert gas, for example helium, argon or other inert gas including mixtures thereof.

The loading is typically performed at a pressure above atmospheric pressure, for example from atmospheric pressure up to a pressure of 10 bar.

The nitric oxide loaded zeolites may be sealed inside airtight packaging for storage and transport purposes.

Upon exposure of the nitric oxide loaded zeolite to moisture, for example an aqueous environment such as water or blood, the nitric oxide is displaced from the metal complex inside the zeolite resulting in release of nitric oxide gas into the aqueous environment.

The nitric oxide may also be released from the nitric oxide loaded zeolite when placed in air.

The release of nitric oxide may occur at a variety of temperatures, however room temperature or body temperature is preferred.

The nitric oxide loaded zeolite may be prepared in the form of a powder or a monolith for use for example in topical therapeutic applications or *in vitro* applications such as delivery of specific amounts of NO to cell cultures. For example, a specific amount of NO may be loaded into a zeolite and then, knowing the extent of release or release profile of the NO loaded zeolite, a precise amount of NO may be delivered to the cell culture. This principle may also be applied to other delivery applications of NO e.g. in therapeutic applications so that a specific amount or dose of NO may be administered.

The monoliths may be formed by compression of a zeolite powder or by mixing a powdered zeolite with a suitable binder which is well known in the manufacture of zeolite catalysts.

Suitable binders include, but are not limited to, ceramic binders e.g. silica or alumina, polymeric binders, e.g. polysulfone, polyethene, PET, polystyrene and other polymers.

Alternatively the zeolites may be provided as coatings on medical devices such as metallic medical devices. The coated devices may then be delivered to the locality where the nitric oxide is required. For

example, a zeolite coated stent may be used to perform balloon angioplasty and the release of nitric oxide under these conditions may be used to reduce restenosis.

Typically, the zeolites are provided in a suitable form as discussed above, and then loaded with nitric oxide ready for storage and use at a later time.

A powdered zeolite loaded with nitric oxide may be used in topical applications such as for wound dressing, and may be provided in a bandage for application to a wound for release of the nitric oxide into the wound to aid healing. A zeolite provided as a monolith may be used e.g. for topical applications or, for example, for suppository application in the treatment of severe constipation.

According to a third aspect of the present invention there is provided a zeolite material comprising releasably adsorbed nitric oxide for use in surgery and/or therapy.

According to a fourth aspect of the present invention there is provided a pharmaceutical preparation comprising a zeolite material comprising releasably adsorbed nitric oxide together with a pharmaceutical carrier.

The present invention also provides the use of a zeolite material comprising releasably adsorbed nitric oxide in the preparation of a medicament for use in the treatment or prophylaxis of disease. Diseases or medical conditions which may be treated include infections of the skin, including dermatophyte fungi, leishmaniasis, molluscum and papilloma virus, and mycobacterium infections. Further uses include therapeutic applications in anti-neoplastic activities, immune response modification, treatment of Raynaud's disease, wound healing and skin pigment modification. Yet further

uses include treatment of restonsis, psoriasis and eczema, and skin cancer (melanoma). Therapies for other bacterial problems include the reduction of severe foot or body odour problems, and in the treatment of Methicillin Resistant Staphylococcus Aureus infections.

According to a sixth aspect of the present invention there is provided a medical article comprising a zeolite material.

The zeolite material of the medical article may be provided without nitric oxide loaded therein to allow loading with nitric oxide prior to use and/or storage of the medical device ready for subsequent use.

Alternatively, the zeolite material of the medical article may be provided as a zeolite material comprising releasably adsorbed nitric oxide.

Suitable medical articles for use in the present invention include a stent, catheters, wound dressings, bandages, self-adhesive plasters and patches.

The present invention also provides, as a seventh aspect, a method of releasing nitric oxide comprising the steps of

(i) providing a zeolite material comprising releasably adsorbed nitric oxide;

(ii) contacting said zeolite material with a medium into which said nitric oxide is to be released.

Such release of nitric oxide is preferably achieved in a controlled manner, for example, by providing a suitable zeolite material with an established controlled release profile.

The medium into which the nitric oxide is to be released may be simply air surrounding the nitric oxide loaded zeolite, or may be, for example, an aqueous medium.

The release may be performed either inside an animal body, topically to an animal body or in non-body applications such as release into cell cultures.

The release may be performed at any suitable temperature, however room or body temperature is preferred.

The method of releasing nitric oxide may be applied to the treatment of humans or animals and accordingly the present invention further provides as an eighth aspect a method of treatment or prophylaxis of an individual in need thereof comprising providing a zeolite comprising releasably adsorbed nitric oxide and contacting said zeolite with said individual.

Embodiments of the present invention shall now be described with reference to the following non-limiting examples in which:

Example 1 describes the preparation of ion-exchanged zeolites;

Example 2 describes the preparation of nitric oxide-loaded zeolites;

Example 3 describes the release of nitric oxide from a nitric oxide loaded zeolite into the atmosphere;

Example 4 describes the release of nitric oxide from a nitric oxide loaded zeolite into solution;

Example 5 describes the release of nitric oxide from an alternative nitric oxide loaded zeolite into the atmosphere;

Example 6 describes the quantification of nitric oxide in solution by direct measurement.

Figure 1 is a graph showing the release profile of NO into the atmosphere according to Example 3.

Figure 2 is a bar chart showing the release profile of NO into the atmosphere at different times according to Example 5.

Figure 3 is a graph which shows the amount of dissolved NO concentration in accordance with Example 6.

Example 1

Preparation of ion-exchanged zeolites

The synthesis of zeolites is well known to those with knowledge of the art, and ion exchange of the zeolites can be carried out by standard methods (Plank et. al., U.S. Patent No. 3,140,249; Preparation, characterisation, and Performance of Fe-ZSM-5 Catalysts R. Joyner and M. Stockenhuber, J. Phys. Chem. B., 1999, 103, 5963-5976). The ion-exchanged zeolite is then dehydrated under vacuum. Analysis of the zeolites is carried out using elemental analysis, X-ray diffraction and spectroscopic analysis.

An example of the preparation of a dehydrated ion-exchanged zeolite is described below.

The zeolite (MFI, 2g) was placed in a 0.05 M solution of the metal ion (200 ml, distilled water) to be exchanged and stirred for 24 hours. Alternatively, with the same concentrations the exchange could be carried out under dry conditions in an inert atmosphere (argon) with sonication using methanol as a solvent. The products were recovered by filtration/centrifuge.

The concentration of the metal ion solution and time for the exchange can be varied to vary loading of the metal into the zeolite. Specific examples of different metal ions that have been loaded into the zeolites are given in Table 1.

Table 1 - Elemental composition of ion-exchanged zeolites prepared using this methodology. The table shows a range of ion exchange behaviour from very low exchange in the case of iron up to over exchange in the case of copper. Initial composition of the zeolite - $(NH_4)_z[Al_zSi_{2-z}O_4]$ where z = 0.13333 (Si/Al = 14)

Cation (M)	Final Al/M ratio
Fe^{3+}	17.82
Ni^{2+}	8.42
Co^{2+}	3.84
Cu^{2+}	1.50

Example 2

Preparation of NO-loaded zeolites

Nitric oxide can be produced in situ, or introduced from a cylinder. An example of the preparation of an NO-loaded zeolite is given below.

A 1M asorbic acid solution (200 ml) was degassed by bubbling argon through the solution with stirring. This was then added dropwise to sodium nitrite (~5g) which had been purged with argon for 30 minutes. A slow flow of argon was used to carry the produced nitric oxide through high surface area potassium hydroxide to remove higher nitrogen oxides, and then through calcium sulfate to dry the gas stream, before being allowed to flow through the ion-exchanged zeolite (e.g., Fe-loaded MFI zeolite ~0.5g) then finally through a bubbler.

The NO-loaded zeolite is then sealed inside the vessel and stored under the Ar/NO atmosphere until required. The same method of NO-loading is used for all the zeolites, independent of framework type and ion exchange.

Example 3

Release of NO into the atmosphere from NO-loaded Fe-MFI zeolite

Thermogravimmetric analysis coupled with mass spectroscopic analysis of the resultant gases was used to study the temperature dependence of the evolution of nitric oxide from the zeolite. The results are reproduced graphically in Figure 1 which shows the profile of weight loss (line A) and ion current (line B) for NO in a mass spectrometer versus temperature. NO-loaded Fe-MFI zeolite (0.010g) was placed in a Netzch Thermogravimmetric analyser coupled to a mass spectrometer. The sample was heated to 300°C at $10°min^{-1}$ 48 hours under flowing air and the gases evolved analysed using mass spectrometry. The resultant trace indicated that the amount of NO released increases up to 130°C before it begins to reduce. However, at ~180°C a sharp spike in NO production is seen, coinciding with a phase transition in the zeolite sample (confirmed by differential scanning calorimetry). This is the well known monoclinic to orthorhombic phase transition that occurs in zeolite MFI. The phase transition temperature can be altered by careful choice of the silicon to aluminium ratio of the starting zeolite, by controlling the ion exchanged cation and amount, and by controlling the amount of NO loading. Thus property can then lead to a tailored NO release, by for example, a heat pad applied to a wound healing bandage - at temperatures below the phase transition NO release is slow, while above the phase transition NO release is much enhanced. Figure 1 shows the phase transition at 180°C, but there are

literature reports of phase transition in zeolite MFI as low as -100°C (H Morell, K Angermund, A R Lewis, DH Brouwer, C A Fyfe, H Gies Structural investigation of Silicate-I loaded with n-hexane by X-ray diffraction, Si-29 MAS NMR, and molecular modeling. *Chem. Mater. 14, 2192, 2002*). The precise transition temperature depends on the composition of the zeolite and the loading of NO. Other zeolites, such as FER also show phase transitions that can be tailored in this way.

Example 4

Release of NO into solution from NO-loaded Fe-MFI zeolite

Fe-MFI nitric oxide adsorbed sample (0.013 g) was placed in distilled water (10.452 ml) was tested for nitrite (Quantofix Nitrite Sticks) which give a positive result with 20 mg/l NO_2. A sample of distilled water was tested for nitrite (as a reference) which resulted in 0 mg/l NO_2. Nitrite is formed in solution from the reaction of NO with water and oxygen and is therefore an indirect method for the measurement of NO in solution.

Example 5

Release of NO into atmosphere from NO-loaded Fe-ZSM-5

A small sample of NO-loaded Fe-ZSM-5 (0.010g) was placed in a Netzch Thermogravimmetric analyser coupled to a mass spectrometer. The sample was heated to 37°C for 48 hours under flowing air and the gases evolved analysed using mass spectrometry. The resultant trace indicated that NO is slowly released from the zeolite at these temperatures into the atmosphere. Figure 2 shows the profile of NO

released from the zeolite at different times during the cycle. The bar chart shows ion current (from mass spectrometer) versus time for four molecules (H_2O, NO, NO_2 and HNO_2) released from NO-loaded Fe-MFI. It can be clearly seen that NO is the most abundant gas given off at all times.

Example 6

Quantification of NO in solution by direct measurement using a nitric oxide electrode.

The World Precision Instruments ISO-NO Mark II nitric oxide electrode was calibrated using the titration method according to the procedure described by World Precision Instruments (ISO-NO Mark II Instruction Manual, World Precision Instruments, 2002). The metal ion-exchanged zeolite with adsorbed nitric oxide was transferred into a glass tube and wet argon (5 ml min^{-1}) was allowed to flow over it. This stream was then directed to bubble through a buffered solution (pH 7.4 at 37°C) into which the nitric oxide electrode was immersed. Data on the release of nitric oxide was then collected over several hours.

Figure 3 shows the dissolved nitric oxide concentration (not normalised for mass of zeolite or degree of ion exchange) produced when three NO-loaded zeolite samples are exposed to a flow of moist argon as described above. The gas flow is then bubbled through the buffered solution and the nitric oxide concentration measured with time. The experiment measures the uptake of nitric oxide by the solution, and takes no account of loss of nitric oxide that does not dissolve in the liquid. However, for many of the proposed applications (e.g. as a wound-

healing bandage) where release of nitric oxide is not directly into a solution, this experiment mimics the situation more closely than would release of the nitric oxide directly into a liquid.

The results illustrate that different nitric oxide-loaded zeolite materials release NO in different ways. Zeolites with the LTA structure release their NO relatively quickly, while those based on the PHI framework release nitric oxide over a much longer timescale. It is noted that the copper and iron ion exchanged LTA zeolites show similar release profiles. The results do show in all cases that the concentration of nitric oxide in the solution is of similar magnitude (nanomolar to micromolar concentrations) to that found in many biological situations.

The examples hereinabove are not to be construed as limiting on the scope of the present invention, but merely representative embodiments thereof. Other ways of performing the invention will be apparent to the skilled person, in particular we refer to APPENDIX A attached hereto which provides further background material and experimental results encompassed by the present invention.

APPENDIX A

The use of zeolite-A to store and deliver the biologically active molecule nitric oxide

Nitric oxide (NO) is the archetypal Jekyll and Hyde molecule. We go to great lengths to remove it from the exhaust gases of automobiles as it is a harmful pollutant contributing to photochemical smog. However, NO also plays an important and positive role in mammalian biology.[1] It is implicated in many processes in the body including vasodilation, the prevention of platelet aggregation/adhesion and thrombus formation, neurotransmission and the immune system. There are tremendous possibilities for the use of NO in prophylactic and therapeutic procedures[2,3] but unfortunately, because NO is active in so many biological processes it needs to be delivered in a targeted manner to avoid adverse side-effects at other sites in the body. As NO is a gas it is difficult to accomplish targeted delivery in a controlled manner and in the right quantities. Until these problems are solved many areas in which NO has the potential to improve the quality of human life will be left unexplored. Zeolites are important nanoporous solids that have been extensively investigated for the removal of nitrogen oxides (DeNOx catalysis) from automotive exhaust gases.[4,5] In this paper we report the use of zeolites, not to destroy NO, but to store and deliver it in amounts of biological and medical significance. We then demonstrate the material's activity in the inhibition of platelet aggregation.

Nitric oxide (NO) shows great possibilities in medicine, with potential applications that include anti-thrombogenic[6,7,8] and antibacterial[9] devices, improved dressings for wounds and ulcers[10,11] and the treatment of bacterial infections.[12] Despite our rapidly increasing knowledge of the beneficial effects of nitric oxide the development of NO-based therapies has been frustratingly slow. A significant barrier to the use of NO in

medicine is the lack of suitable delivery mechanisms. Systemic delivery of nitric oxide to the body is possible through drugs[13] that produce NO after metabolism (such as glyceryl trinitrate, which is used to manage the symptoms of angina) or that release NO spontaneously on contact with physiological fluids, (such as the so-called NONOate series of compounds[2] or nitrosothiols[14]). However, the first displays tolerance because of the need for metabolism and the others, when delivered directly into the blood stream may cause dangerous lowering of systemic blood pressure. The immobilisation of NO-releasing compounds in polymer supports[6,7,8] and the use of two component creams that produce NO after mixing,[15] are methods for targeted delivery of NO that have been proposed for use in the body and on the skin respectively. These methods show promise for the manufacture of anti-thrombogenic medical devices,[6,7,8] for the alleviation of abnormal vasoconstriction in the blood supply of the skin (Raynaud's syndrome)[15] and for wound healing.[10] However, they both have their problems. The supported polymers are often prepared in multi-step processes and require loading of NO under high pressure. A number of them are also unstable at room temperature and must be stored cold. The two-component creams release a short-lived burst of nitric oxide on acidification of nitrite. This takes place at low pH and is a process unsuitable for medical application, especially on patients with sensitive skins. There is therefore a great need for new materials that can store and deliver suitable amounts of nitric oxide without such problems. The requirements for a NO-releasing material that can be used in biological/medical applications are that the material can deliver the required amount of NO and that the gas should be released by a simple activation process, producing no side products. The material should also be of low toxicity, easy to handle and preferably relatively simple to prepare and low cost. We show here that zeolite-A fits all these

criteria, adsorbing NO and then releasing it on contact with water in amounts that are biologically significant over a period of several hours.

Zeolites have well known applications in ion exchange, gas adsorption and catalysis (including DeNOx for removal of nitrogen oxides from automotive exhaust gases), although they are of increasing interest as hosts for nanotechnology applications.[16] New zeolites[17] and new methods for zeolite preparation[18] continue to attract attention in catalysis, but their use in biomedical applications is limited to non-toxic medical diagnosis tools.[16] Zeolite-A (given the three letter framework code LTA[19]) is a very well known material, manufactured in greater than 1M tonne amounts annually for use as a detergent builder and water softener. The structure of zeolite-A[20,21] consists of alternating SiO_4 and AlO_4 tetrahedra that share corners to produce the open framework depicted in Figure 1, with ion exchangeable cations residing in the channels of the structure. It is well known for its affinity to water, often being used (under the name Molecular Sieve 3A, 4A or 5A) to dry solvents in organic chemistry.

Samples of zeolite-A were synthesised according to the procedure given in the *Verified Syntheses of Zeolitic Materials.*[22] Standard ion-exchange procedures were then used to replace the sodium ions in the as-made form with various transition metal cations that are known from DeNOx studies to bind nitric oxide strongly (Mn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}). The transition metal zeolite A samples were then dehydrated to remove water, exposed to dry nitric oxide and stored in sealed Schlenk tubes at room temperature ready for use. Chemical analysis of the zeolites are given in the supplementary material.

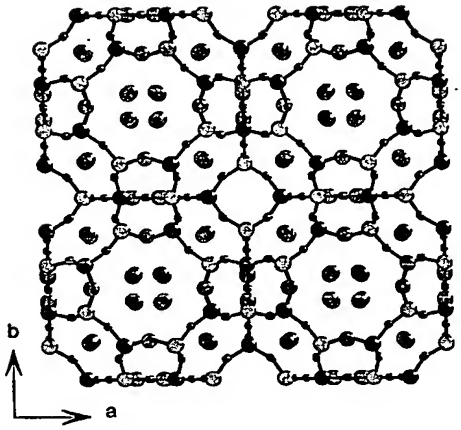

Figure 1. The crystal structure of dehydrated Na-zeolite-A. The structure consists of alternating Si (blue) and Al (purple) centred tetrahedra with sodium cations (green) bound to the oxygen atoms (red) of the framework. The sodium cations can be readily exchanged with transition metal ions. For clarity, only the Al-O and Si-O bonds are drawn. Data taken from reference 20.

We are particularly interested in designing materials to deliver NO above chronic wounds, as animal models have shown that topical application of NO can significantly promote wound closure[10] and there is evidence that NO can be used to treat diabetic ulcers.[11] The best model for this is the release of NO into a moist atmosphere that is in contact with the liquid phase (phosphate buffered saline pH 7.4). The amount of nitric oxide adsorbed by the solution is then measured using a nitric oxide electrode. Figure 2 shows the NO release profiles measured in this way for a number of transition metal exchanged zeolite-A samples in contact with an argon flow that has been saturated with water vapour. The order of how much NO is released for each different metal agrees well with the NO adsorption properties of transition metal zeolites in pressure swing adsorption studies,[23] Co-exchanged zeolites releasing the most NO while the original sodium form of the zeolite releases the least NO. At first glance the copper-exchanged

zeolite-A results seem anomalously low especially since Cu-zeolites are well known deNOx catalysts. This is because the zeolite is overexchanged, with more copper ions in the channels than is strictly necessary for charge balance reasons. Many of the 'extra' copper ions are probably present as hydroxide species[4] and so reduce the availability of the metal ions for NO coordination. The cross-over of the Mn^{2+} and Ni^{2+} exchanged zeolites may indicate different distributions of the metal ions between the three possible extraframework cations sites in zeolite-A, some of which may be more susceptible to substitution by water than others.

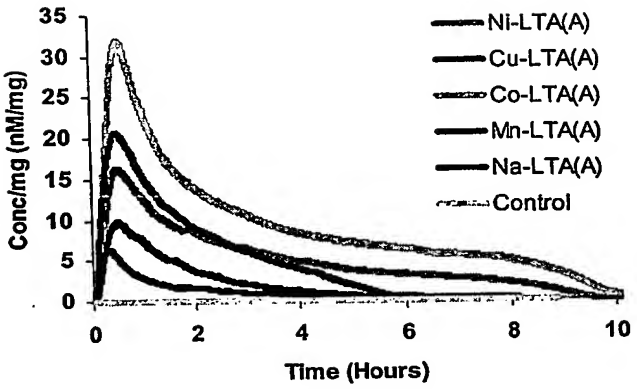

Figure 2. Nitric oxide release profiles of NO-loaded metal-exchanged zeolite-A. The control is a Co^{2+}-exchanged zeolite that has not been exposed to nitric oxide. The electrode response results have been normalised to give the concentration of NO in solution per mg of zeolite material.

Importantly, the release of NO takes place over a relatively long period of time (~10 hours in Figure 2), and if there is less water vapour present the release takes place over an even longer time period. Figure 3 shows the release profile of Co- and Mn-exchanged zeolite A into both 'wet' (water vapour saturated) and 'almost-dry' argon atmospheres, in the latter case the zeolites still gave off measurable amounts of nitric oxide more than 24 hours after the experiment began. This shows the importance of water in the mechanism of the NO release from these zeolites.

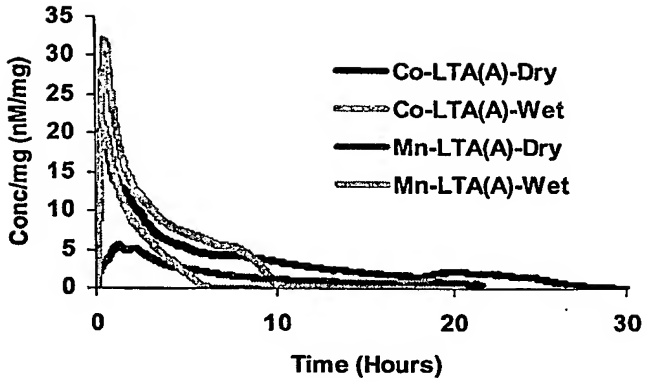

Figure 3. The influence of moisture content on NO-release profiles. The NO-release profiles of Co- and Mn- exchanged zeolite A into both 'wet' (water vapour saturated) and 'dry' argon atmospheres. In the 'wet' experiments the argon was bubbled through hot (80°C) deionised water prior to contacting the zeolite. In the dry experiment the argon was taken directly from the gas cylinder and partially dried over calcium sulphate.

The amount of nitric oxide released by the zeolite depends not only on which transition metal is present but also on how much of a particular metal is present. Zeolite-ZK4 is a variant of zeolite-A that has the same framework structure and so has the same framework code (LTA). However, there are fewer exchangeable cations in zeolite-ZK4 as there is aluminium in the framework. This means that there are fewer metal cation sites in the channels of the structure to bind nitric oxide. It can be clearly seen in Figure 4 that Co-exchanged zeolite-A releases more NO than Co-exchanged zeolite ZK4, consistent with the reduced level of cobalt in the ZK4 structure.

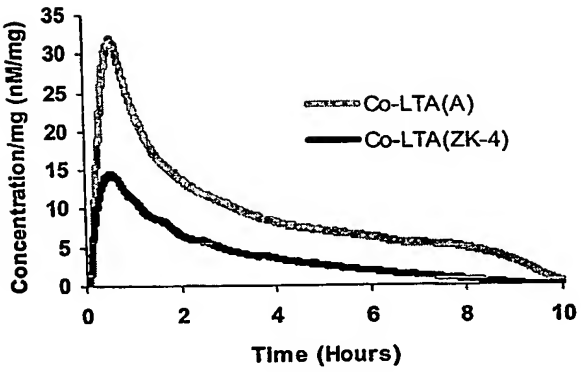

Figure 4. Dependence of NO-release on Co^{2+} exchange level. Co-zeolite-A (CO-LTA(A)) contains %wt cobalt, while Co-zeolite-ZK4 (Co-LTA(ZK4)) contains %wt cobalt.

The above experiments indicate the potential of NO-loaded zeolites to deliver nitric oxide into a moist atmosphere for delivery above the skin for applications such as the promotion of wound healing, the treatment of diabetic ulceration or the prevention of bacterial infection. They also illustrate the controllable nature of the NO delivery, which can be changed by varying the type and amount of transition metal present in the zeolite structure. However, the potential applications of NO-releasing materials do not end here. The need for improvements in the biocompatibility of materials is a very important target. This is particularly true for blood contacting solids that are used in vascular grafts and extracorporeal tubing that is necessary in coronary bypass surgery. Life-threatening complications can occur if thrombosis formation (platelet aggregation and adhesion) is induced by materials that are in contact with blood.[7] Thrombus formation in healthy circulatory systems is inhibited in a number of ways, including the production of small quantities (approximately 1 pmol min^{-1} mm^{-2}) of NO by the endothelial cells that line the blood vessels and by blood platelets. A potentially important strategy for reducing post-operative complications is to make medical devices out of an NO-releasing material, thereby mimicking the action of the endothelial cells.

The Co-exchanged zeolite samples, in a 75:25 wt % mixture with powdered polytetrafluoroethylene (PTFE) were prepared as mechanically stable pressed disks and subsequently dehydrated and loaded with nitric oxide in the same way as the powdered samples. Tests with disks made from only NO-exposed PTFE showed no delivery of nitric oxide. The zeolite/PTFE disks were then suspended in a steel wire holder below the surface of platelet rich plasma (PRP) in the cuvette of a four-channel platelet aggregometer at 37°C. After a short induction period (1 minute), platelet aggregation was initiated and then measured as a change in turbidity (light transmission) of PRP against a platelet poor plasma (PPP) blank. The results (Figure 5) show that a NO-loaded Co-exchanged zeolite-A/PTFE sample completely inhibits platelet aggregation while a Co-exchanged zeolite/PTFE sample that has not been loaded with NO shows no inhibition of aggregation when compared to a PRP control where no zeolite or PTFE was added. This experiment illustrates well the potential of the NO-loaded zeolite-A to inhibit thrombosis in physiological solutions and the possibilities of using the zeolites as NO-releasing components in medical devices, perhaps when blended with polymers such as PTFE.

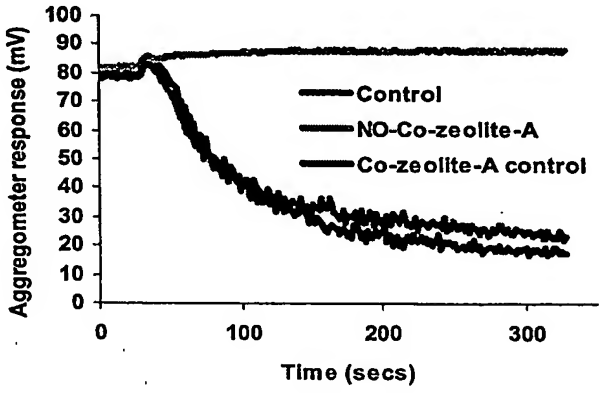

Figure 5 Platelet aggregation experiments. The control (only PRP) and Co-zeolite-A control (PRP + Co-zeolite-A/PTFE; no nitric oxide loading) show

essentially the same aggregation behaviour while the NO-loaded Co-zeolite-A shows 100% inhibition of platelet aggregation.

Gas storage by porous materials is currently an important topic, but tends to concentrate on the storage of gases for energy applications.[24] We have demonstrated that zeolites have great potential as NO storage and release materials for biological and medical applications. Their preparation and loading with nitric oxide is relatively facile, and they are stable when stored in anhydrous conditions at room temperature. The delivery of NO occurs by simple reaction with water, and the amount of nitric oxide released can be tailored by altering both the type and number of metal cations in the structures. We have also demonstrated that NO-releasing zeolites inhibit platelet aggregation in physiological fluids, a potentially important application of such solids in the prevention of thrombus. The work overturns the conventional idea that zeolites can be used only to destroy nitric oxide and adds a new delivery system to the armament of researchers developing NO-based therapies.

Methods

Ion exchange of zeolite-A.

Metal ion-exchanged zeolites were prepared as follows. Typically, the as-synthesised sodium zeolite-A (5 g) was placed in a 0.05 M solution of the metal acetate (400 ml, distilled water) and stirred for 24 hours. The products were recovered by filtration, washed with distilled water (400 ml) and dried at 100 °C overnight. Elemental analysis was carried out to determine the chemical composition of the zeolites using an Agilent 7500 Series ICP-MS spectrometer.

NO loading and release experiments

The ion-exchanged zeolite (~0.3 g) was dehydrated for 2 hours at 300 °C *in vacuo* (0.5 mm Hg). This was cooled to room temperature and exposed to approximately 3 atm of a nitric oxide/helium gas mixture (10 % NO, 90 % He) for 10 minutes, evacuated and exposed again to 3 atm of nitric oxide. This was repeated three times.

For the measurement of NO-release, a flow of argon (either saturated with water vapour or taken directly from the gas cylinder, 5 ml min^{-1}) was passed over a known amount of the NO-loaded zeolite. The gas was then bubbled through phosphate buffered saline solution (pH 7.4, 10 ml) in which a previously calibrated nitric oxide electrode (World Precision Instruments, ISO-NO Mark II) was immersed. The concentration of NO was measured over the course of several hours. All experiments were repeated three times and gave reproducible results.

Platelet Aggregation

The zeolite was ground with PTFE to in the desired ratio (75% zeolite/ 25% PTFE). The mixture was then pressed into disks (5 mm, ~ 20 mg) under 2 tons for 30 seconds. The disks were then dehydrated and loaded with nitric oxide in the same way as the powder samples.

Venous blood was drawn from the antecubital fossa of healthy volunteers (aged 20-40 years) into citrated tubes (0.38% final concentration). Volunteers had not taken any medication known to affect platelet aggregation within the last 10 days. Platelet rich plasma (PRP) was obtained from whole blood by centrifugation (350*g*; 20min; room temperature). Platelet poor plasma (PPP) was obtained by further centrifugation of PRP (1200*g*; 5min; room temperature).

The zeolite/PTFE disks were then suspended in a steel wire holder below the surface of the PRP in the aggregometer cuvette, ensuring that they did not interfere with the light beam or the mechanical stirring (1000 rpm) of the PRP. After a short induction period (1 minute), platelet aggregation was initiated by addition of 8μM U46619 (a TxA2 analogue). Aggregation was measured as a change in turbidity (light transmission) of PRP against a PPP blank.

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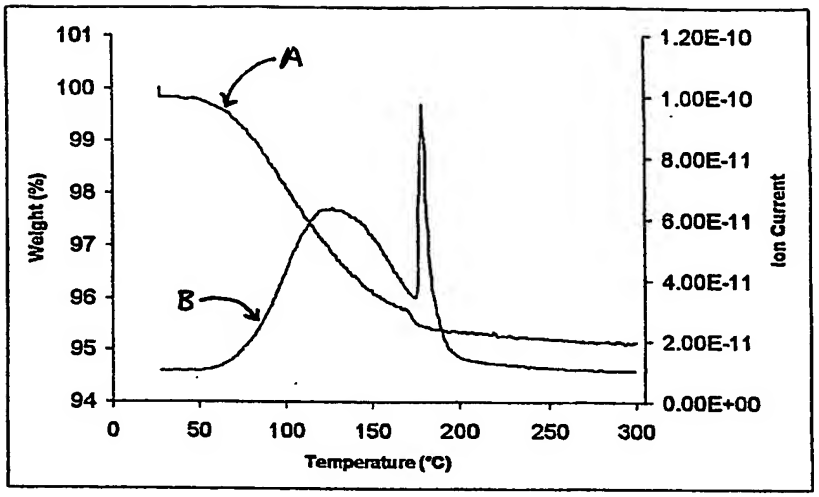


Figure 1

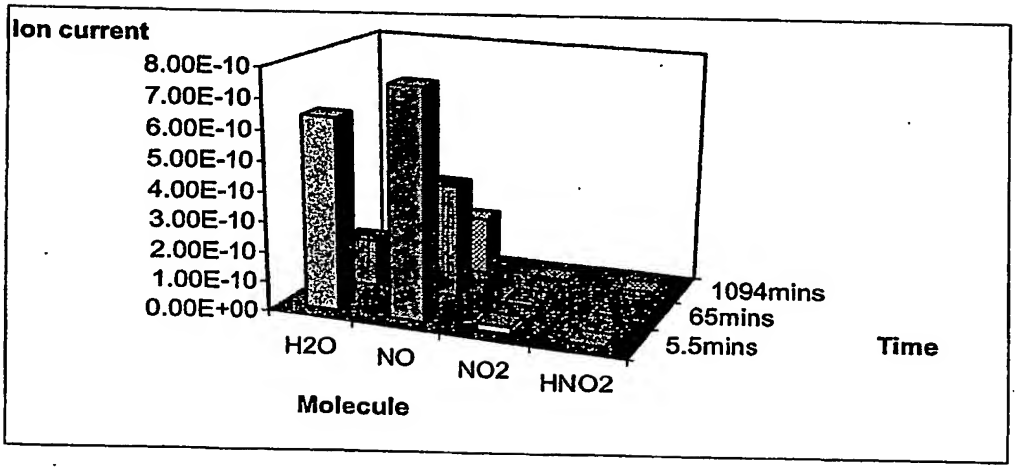
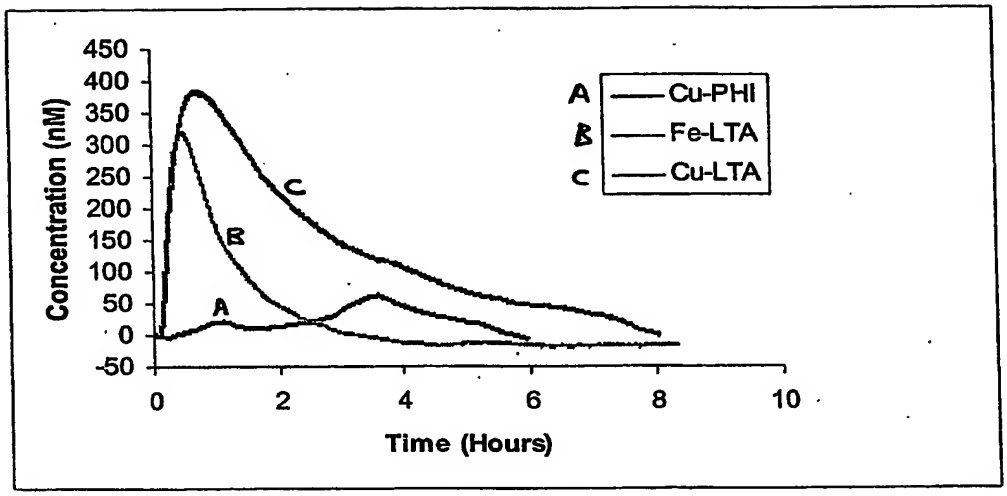


Figure 2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A Cu-PHI
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C Cu-LTA

Figure 3